AD_____

Award Number: DAMD17-98-1-8247

TITLE: Interactions Among Brca1, Brca2, and Components of the Recombination Machinery

PRINCIPAL INVESTIGATOR: Patrick M. W. Sung, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health Sciences Center at San Antonio
San Antonio, Texas 78229-3900

REPORT DATE: June 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual Summary (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Interactions Among Brca1, Brca2, and Components of the Recombination Machinery

5. FUNDING NUMBERS
DAMD17-98-1-8247

6. AUTHOR(S)
Patrick M. W. Sung, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas Health Sciences Center at San Antonio
San Antonio, Texas 78229-3900

E-Mail: sung@uthscsa.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

DNA double-strand breaks are induced by endogenous free radicals and environmental agents such as ionizing radiation. The repair of DNA double-strand breaks is important for preventing possible chromosomal fragmentation, translocations, and deletioins induced by these breaks. The accurate repair of Dna double-strand breaks is mediated by a group of genes called the *RAD52* epistasis group and proceeds via a recombinational mechanism. In mammals, the efficiency of recombinational DNA repair is modulated by the tumor suppressors *BRCA1* and *BRCA2*, providing compelling evidence that this DNA repair pathway functions to suppress cancer formation. Importantly, recombinational DNA repair is also required for the removal of interstrand DNA crosslinks formed by bifunctional crosslinking agents, which are commonly used to treat various malignancies. Our research efforts are directed at establishing biochemical models for examining the functions of the various *RAD52* group components and for delineating the mechanism of recombinational DNA repair. Recent studies in our laboratory have established an in vitro system for examining the recombinase activity of humas Rad51 and defining the role of various recombination factors in influencing this Rad51 activity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

DNA double-strand breaks (DSBs) are induced by ionizing radiation and arise during meiosis and replication of a damaged DNA template. If not handled properly, DSBs often lead to gross chromosome rearrangements. Homologous recombination is a major means for the repair of DSBs in all eukaryotes. Importantly, there is compelling evidence that homology-directed recombinational DNA repair is needed for breast cancer avoidance in humans.

In homologous recombination, the ends of the DSBs are subjected to exonucleolytic processing, which results in the formation of 3' single-strand DNA tails a few hundred bases in length. Nucleation of the recombination factors onto these single-strand tails renders them recombinogenic, leading to a search for the chromosomal homolog and the stable pairing of the single-strand tails with the homolog to form a DNA joint called "D-loop". In the later stages, DNA synthesis occurs, followed by the resolution of DNA intermediates to yield mature recombinants and restore the integrity of the injured chromosome. Extensive genetic evidence indicates that the evolutionarily conserved genes of the *RAD52* epistasis group, of which *RAD54* and *RAD54B* are key members, mediate D-loop formation. Currently, there is a paucity of information on the functions of the Rad54 and Rad54B proteins in the recombination reaction. We have cloned both the *RAD54* and *RAD54B* genes from a human testis cDNA library and have expressed their encoded products in insect cells using recombinant baculoviruses. Rad54 protein has been purified to near homogeneity and found to possess a DNA supercoiling function that is driven by ATP hydrolysis. Importantly, we find that Rad54 protein functionally and physically interacts with the Rad51 recombinase. Further studies are proposed to delineate the action mechanism of Rad54 and Rad54B proteins in the Rad51-mediated D-loop reaction, and also to purify and define the functions of higher order complexes containing these proteins. These studies should shed some light on the biochemical functions of Rad54 and Rad54B and should also provide a biochemical model for dissecting the mechanism of homology-directed DNA repair in human cells.

BODY

In eukaryotic organisms, the repair of DNA double-stranded breaks by homologous recombination is mediated by a group of evolutionarily conserved genes known as the RAD52 epistasis group, of which, Rad51, Rad54, Rad54B are key members (reviewed by Sung et al, 2000). In mammals, the efficiency of homology-directed DNA repair is regulated by the tumor suppressors BRCA1 and BRCA2 (Pierce et al, 2001), thus providing a compelling link between this DNA repair pathway and the suppression of cancer formation. The involvement of the homologous recombination machinery in the maintenance of genome stability and tumor suppression underscores the need for deciphering the action mechanism of this machinery.

During the homologous repair of DNA double-stranded breaks, a single-stranded DNA intermediate is utilized by the recombination machinery to invade a DNA homolog, most often the sister chromatid, to form a DNA joint molecule referred to as a D-loop (Sung et al, 2000; Figure 1). D-loop formation is critical for subsequent steps in the recombination reaction, which include repair DNA synthesis and resolution of recombination intermediates (Sung et al, 2000), that lead to the restoration of the integrity of the injured chromosome.

Rad51 is the structural and functional homolog of the E. coli general recombinase enzyme RecA (Sung, 1994; Sung et al, 2000). The RAD54 and RAD54B encoded products belong to the Swi2/Snf2 protein family and appear to be unique to eukaryotes (Sung et al, 2000). Purified Rad54 has DNA-dependent ATPase and DNA supercoiling activities. However, the manner in

which Rad54 influences the Rad51-mediated D-loop reaction has remained mysterious. We have been conducting studies aimed at dissecting the biochemical functions of Rad54 and Rad54B proteins and their interactions with Rad51 in recombination and DNA repair.

Purification of human recombination factors- Human Rad51 protein was expressed in E. coli and purified to near homogeneity (Fig. 1A) as described previously (Sigurdsson et al, 2001). Full length human RAD54 and RAD54B cDNAs were cloned from a testis cDNA library using the polymerase chain reaction. The cloned cDNAs were sequenced to ensure that they agree with the published sequences. We tagged Rad54 with a FLAG epitope and expressed it in insect cells by the use of a recombinant baculovirus. We obtained ~1 mg of nearly homogeneous Rad54 protein (Fig. 1B) from 500 ml of insect cell culture by a combination of conventional column chromatography and affinity binding to an antibody specific for the FLAG epitope. Untagged Rad54B has been expressed in insect cells as well (Fig. 1C). We are in the process of devising a procedure for the purification of Rad54B.

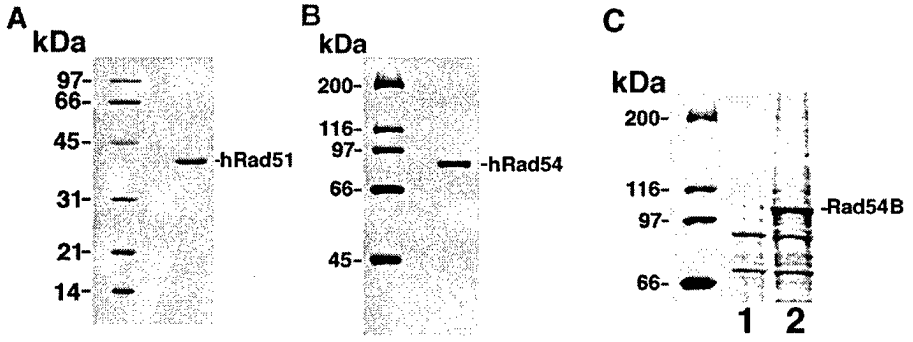

Figure 1. Recombination factors. Purified human Rad51 (A) and human Rad54 (B) were analyzed by SDS-PAGE and staining with Coomassie Blue. In (C), extracts from uninfected insect cells (lane 1) or insect cells infected with the recombinant baculovirus (lane 2) that expresses human Rad54B.

Rad54 physically interacts with hRad51 – Rad51 and Rad54 interact in yeast two-hybrid studies (Golub et al, 1997). To examine whether purified Rad54 physically interacts with Rad51, we coupled Rad51 to Affi-gel beads to use as affinity matrix for binding Rad54. As shown in Figure 2, purified Rad54 was retained on Affi-Rad51 beads but not on the control Affi-beads that contained bovine serum albumin. The results thus indicate a direct and specific interaction between Rad51 and Rad54.

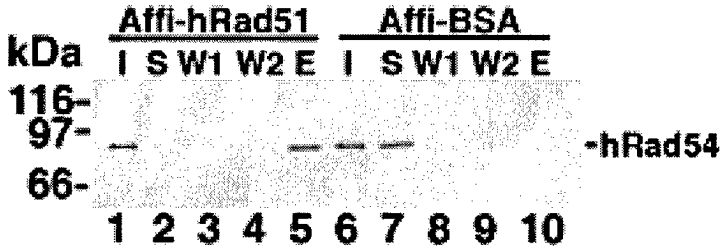

Figure 2. hRad54 interacts with hRad51. Purified hRad54 was mixed with Affi-beads containing either BSA (Affi-BSA) or hRad51 (Affi-Rad51), washed twice with buffer, followed by treatment of the beads with SDS to elute bound hRad54. The starting material (I), supernatant (S), the two washes (W1 and W2), and the SDS eluate (E) were subjected to immunoblotting to determine their hRad54 content.

Rad54 supercoils DNA and promotes separation of DNA strands – We and others have shown that members of the Swi2/Snf2 protein family track on DNA, generating a positively supercoiled domain ahead of protein movement and compensatory negative supercoils behind (Van Komen et al, 2000; Havas et al, 2000; Ristic et al, 2001; see Fig. 3A). As shown in

Figure 3B, purified Rad54 also supercoils DNA in a similar manner, as revealed by treatment of the Rad54-DNA nucleoprotein complex with E. coli topoisomerase I, which removes the negative supercoils, resulting in the accumulation of positive supercoils and formation of an overwound species called Form OW. The negative supercoils produced by Rad54 (Fig. 3A) leads to transient DNA strand opening, rendering the relaxed DNA template sensitive to the single-strand specific nuclease P1 (Fig. 3C). Both the supercoiling and strand opening functions require ATP hydrolysis, as revealed by the omission of ATP or its substitution by the non-hydrolyzable ATP analogues ATP-γ-S and AMP-PNP (Fig. 3, B & C).

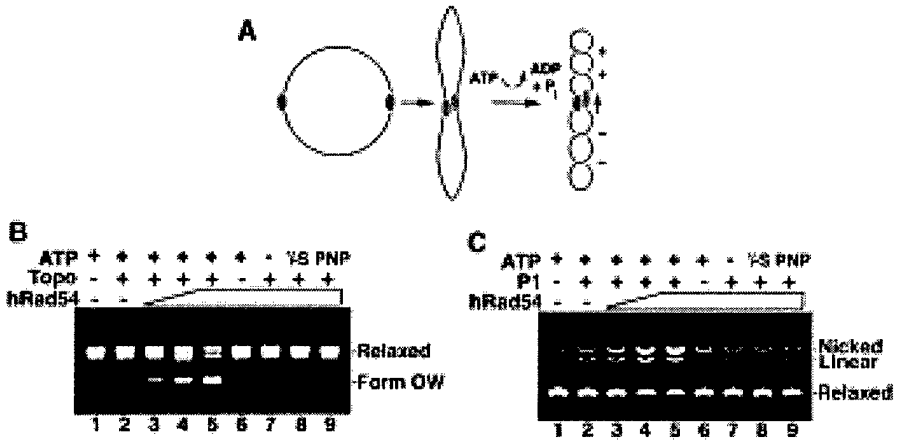

Figure 3. hRad54 supercoils DNA and promotes DNA strand opening. *(A)* Basis for hRad54 induced supercoiling, as per Ristic et al (2001) and Van Komen et al (2000). The free energy from ATP hydrolysis fuels the tracking of a hRad54 oligomer on DNA, producing a positively supercoiled domain ahead of protein movement and a negatively supercoiled domain behind. *(B)* Increasing amounts of hRad54 (200, 400, and 750 nM in lanes 3 to 5, respectively) was incubated with topologically relaxed DNA (20 μM nucleotides) and *E. coli* topoiosmerase I in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of topoisomerase (lane 6) and in the presence of topoisomerase but with the omission of ATP (lane 7) or the substitution of ATP by ATP-γ-S (γ-S; lane 8) and AMP-PNP (PNP; lane 9). DNA alone (lane 1) or DNA incubated with topoisomerase (lane 2) were also included. The reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to reveal the DNA species.
(C) Increasing amounts of hRad54 (200, 400, and 750 nM in lanes 3 to 5, respectively) was incubated with topologically relaxed DNA (20 μM nucleotides) and P1 nuclease in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of P1 (lane 6) and in the presence of P1 but with the omission of ATP (lane 7) or the substitution of ATP by ATP-γ-S (γ-S; lane 8) and AMP-PNP (PNP; lane 9). DNA alone (lane 1) and DNA incubated with P1 in the absence of hRad54 (lane 2) were also included. The reaction mixtures were run in a 1% agarose gel containing 10 μM ethidium bromide.

Rad54 activities are enhanced via interaction with Rad51 – Since Rad54 physically interacts with Rad51 (Fig. 3), we asked whether the DNA supercoiling activity of Rad54 would be enhanced by Rad51. The results showed that hRad51 greatly stimulates the supercoiling reaction, as indicated by a much higher level of Form OW DNA (Fig. 4A). In the P1 assay that measures DNA strand opening, the inclusion of Rad51 markedly increased the nicking of the relaxed DNA substrate (Fig. 4B). Rad51 alone is devoid of DNA supercoiling or strand opening activity (Fig. 4, A & B). Even with the inclusion of Rad51, no Form OW DNA or nicking of DNA was seen when ATP was omitted or substituted by the non-hydrolyzable analogues ATP-γ-S and AMP-PNP (Fig. 4). Thus, the results revealed that Rad51 stimulates the ability of Rad54 to supercoil DNA and unwind DNA strands.

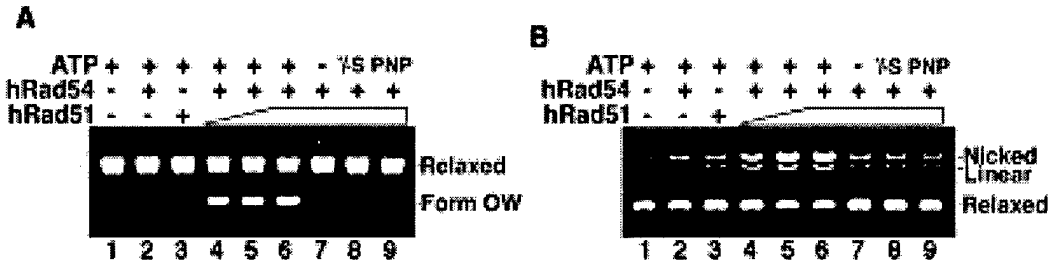

Figure 4. The hRad54 activities are stimulated by hRad51. *(A)* Relaxed DNA (20 μM nucleotides) was incubated with hRad54 (75 nM in lanes 2, and 4 to 9) and hRad51 (80, 160, and 240 nM in lanes 4 to 6, respectively) in the presence of ATP and *E. coli* topoisomerase I. The highest amount of hRad51 (240 nM) was incubated with substrate and topoisomerase I but without hRad54 (lane 3) and also with hRad54 (75 nM) but with the omission of ATP (lane 7) or its substitution with ATP-γ-S (γ-S; lane 8) or AMP-PNP (PNP; lane 9). DNA alone was analyzed in lane 1. After deproteinization, the reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to stain the DNA species.
(B) Relaxed DNA was incubated with hRad54 (75 nM in lanes 2 and 4 to 9) and hRad51 (80, 160, and 240 nM in lanes 4 to 6, respectively) in the presence of ATP and P1 nuclease. The highest amount of hRad51 (240 nM) was incubated with substrate and P1 but without hRad54 (lane 3) and also with hRad54 (75 nM) but with the omission of ATP (lane 7) or its substitution with ATP-γ-S (γ-S; lane 8) or AMP-PNP (PNP; lane 9). DNA alone was run in lane 1. Analysis was carried out in a 1% agarose gel that contained 10 μM ethidium bromide.

Homologous pairing by Rad51 and Rad54 – Genetic studies have implicated Rad54 in promoting the Rad51-catalyzed homolgous DNA pairing reaction that is central to all recombination processes. We employed a well characterized in vitro system (Sung et al, 2000) to investigate a possible functional interaction between Rad51 and Rad54. This assay monitors the incorporation of a ^{32}P-labeled oligonucleotide into a homologous supercoiled target to give a D-loop structure (Fig. 5A). As reported before (Mazin et al, 2000a) and reiterated here (Fig. 5B), hRad51 by itself is not particularly adept at forming D-loop. Importantly, the inclusion of hRad54 rendered D-loop formation possible. D-loop formation by the combination of hRad51 and hRad54 requires ATP hydrolysis, as no D-loop was seen when ATP was omitted or when it was replaced by either ATP-γ-S or AMP-PNP (Fig. 5B). Significantly, the time course revealed a cycle of rapid formation and disruption of D-loop, such that the D-loop level reached its maximum by 1 min but declined rapidly thereafter (Fig. 5B). In fact, by the reaction endpoint of 6 min, little or no D-loop remained (Fig. 5B). Such a cycle of D-loop synthesis and reversal has also been observed for *E. coli* RecA (Shibata et al, 1982), which is due to disassembly of the RecA-ssDNA nucleoprotein complex upon ATP hydrolysis (Bianco et al, 1998).

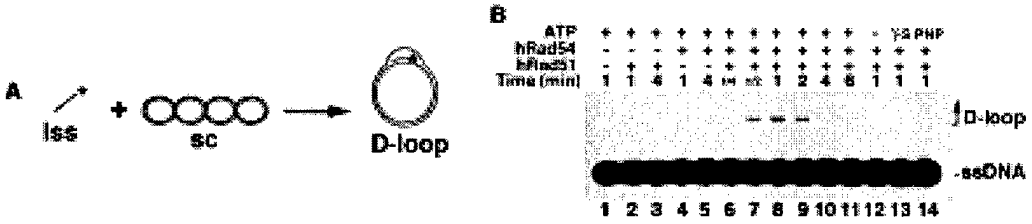

Figure 5. D-loop formation by hRad51 and hRad54. *(A)* Schematic of assay. A radiolabeled 90-mer DNA pairs with a homologous duplex target to yield a D-loop. *(B)* hRad51 alone (lanes 2 and 3), hRad54 alone (lanes 4 and 5), and the combination of hRad51 and hRad54 (lanes 6 to 14) were incubated at 30°C for the indicated times with the DNA substrates in the presence of ATP (lanes 2 to 11), ATP-γ-S (γS; lane 13), AMP-PNP (PNP; lane 14), or in the absence of nucleotide (lane 12). In lane 1, the DNA substrates were incubated in buffer without protein. The protein and DNA concentrations were: hRad51, 800 nM; hRad54, 120 nM; 90-mer oligonucleotide, 2.5 μM nucleotides or 27.7 nM oligonucleotide; pBluescript supercoiled DNA, 35 μM base pairs or 11.6 nM of plasmid.

Higher order protein complexes of Rad54 and Rad54B – Members of the Swi2/Snf2 protein family tend to be associated with other protein factors in higher order complexes that possess chromatin remodeling activities (Vignali et al, 2000). We are most interested in finding out whether Rad54, a member of this protein family, is also present in higher order complexes, we will define the biochemical functions of these complexes. To approach this question, we are in the process of subjecting nuclear extract from Hela and Raji cells to chromatographic fractionation and will identify Rad54 by immunoblotting with highly specific polyclonal antibodies that we already have available. We will employ a variety of tests, including immunoprecipitation and sizing in gel filtration columns or glycerol gradient sedimentation to see if Rad54 from cell extract is found in high molecular weight complexes (purified Rad54 is monomeric in solution). We will also probe the column fractions for Rad54B protein to see if it is part of these complexes. Importantly, Rad54/Rad54B-containing protein complexes will be purified to near homogeneity to enable us to define the novel components by mass spectrometry and to conduct various biochemical experiments including D-loop reaction and chromatin remodeling to delineate the possible functions of these complexes. These biochemical endeavors will be complemented by the appropriate genetic studies in future.

Purification & Characterization of Rad54B - As mentioned above, we already have Rad54B expressed in the baculovirus system (Fig. 1C), and we are in the process of devising a procedure for its purification. When available, we will characterize Rad54B for ATP binding and hydrolysis with or without a ssDNA or dsDNA co-factor, the ability to supercoil and unwind DNA, and physical and functional interactions with Rad51, following the strategies we have used for the characterization of Rad54 protein.

BRCA2 protein - We have expanded considerable effort to express full length human BRCA2 protein in the baculovirus system and have thus far obtained only a small amount of soluble protein. We are currently exploring the use of yeast cells for BRCA2 expression.

APPENDED INFORMATION

(1) Key research Accomplishments to date

- Established procedure for the purification of the human Rad50/Mre11/NBS1 complex from nuclear extracts, and extensive biochemical characterization of this complex for activities germane for homologous recombination and DNA repair.

- Purification of human Rad51 and RPA and demonstration of functional cooperation between these two factors in the homologous DNA pairing reaction central for the formation of heteroduplex DNA joints during homologous recombination and DNA repair.

- Expression and purification of human Rad51B-Rad51C complex and extensive characterization of its DNA binding and ATPase activities and also its functional interactions with human Rad51 in the homologous DNA pairing reaction.

- Expression and purification of human Rad54 and extensive characterization of its ATPase and DNA supercoiling activities and also its functional interactions with human Rad51 in DNA supercoiling and the homologous DNA pairing reaction.

- Expression and purification of human XRCC2 and Rad54B proteins.

(2) Reportable Outcomes

- Invited Speaker, Third International Conference on DNA Repair, Replication, and Recombination, Osaka, Japan. Title of Presentation: "An Overview of the Enzymology of Recombination and DSB Repair in Eukaryotes."

- Invited Speaker, Keystone Conference on Mechanism of DNA Replication and Recombination. Title of Presentation "Recombination Factors that Influence the Rad51 Recombinase Activity".

- Invited Speaker & Session Chair, EMBO Workshop on Mechanisms of Genetic Recombination. Chaired session on "Recombination Proteins". Title of Presentation: "Action Mechanism of Yeast and Human Rad54".

- Invited Speaker, Annual Meeting of the Environmental Mutagen Society, Anchorage, Alaska. Title of Presentation "Functional Interactions among Human Rad51, RPA, and Mediator Proteins".

- Invited Speaker, Radiation Research Symposium in Reno, Nevada. Title of Presentation "Mechanisms and Assembly of Homologous Recombination Protein Complexes".

BIBLIOGRAPHY

Baumann, P., Benson, F.E., West, S.C. 1996. Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. Cell 15: 757-766.

Essers, J., Hendriks, R.W., Swagemakers, S.M., Troelstra, C., de Wit, J., Bootsma, D., Hoeijmakers, J.H., and Kanaar, R. 1997. Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. Cell 89: 195-204.

Golub, E.I., Kovalenko, O.V., Gupta, R.C., Ward, D.C., and Radding C.M. 1997. Interaction of human recombination proteins Rad51 and Rad54. Nucleic Acids Res. 25: 4106-4110.

Gupta, R.C., Bazemore, L.R., Golub, E.I., Radding, C.M. 1997. Activities of human recombination protein Rad51. Proc. Natl. Acad. Sci. U. S. A. 94: 463-468.

Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P.A., Lilley, D.M., and Owen-Hughes, T. 2000. Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. Cell 103: 1133-1142.

Kraus, E., Leung, W.Y., and Haber, J.E. 2001. Break-induced replication: a review and an example in budding yeast. Proc. Natl. Acad. Sci. U S A. 98: 8255-8262.

Krejci, L., Damborsky, J., Thomsen, B., Duno, M., and Bendixen, C. 2001. Molecular dissection of interactions between Rad51 and members of the recombination-repair group. Mol. Cell. Biol. 21: 966-976.

Morrison, C., Shinohara, A., Sonoda, E., Yamaguchi-Iwai, Y., Takata, M., Weichselbaum, R.R., and Takeda, S. 1999. The essential functions of human Rad51 are independent of ATP hydrolysis. Mol. Cell. Biol. 19: 6891-6897.

Pierce, A.J., Stark, J.M., Araujo, F.D., Moynahan, M.E., Berwick, M., and Jasin, M. 2001. Double-strand breaks and tumorigenesis. Trends Cell Biol. 11: S52-59.

Ristic, D., Wyman, C., Paulusma, C., and Kanaar, R. 2001. The architecture of the human Rad54-DNA complex provides evidence for protein translocation along DNA. Proc. Natl. Acad. Sci. U.S.A. 84: 8454-8460.

Sigurdsson, S., Trujillo, K., Song, B-W., Stratton, S., and Sung P. 2001a. Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J. Biol. Chem. 276: 8798-8806.

Shibata, T., Ohtani, T., Chang, P. K., and Ando, T. 1982. Role of superhelicity in homologous pairing of DNA molecules promoted by Escherichia coli recA protein.
J Biol Chem. 257:370-376.

Sung, P. 1994. Catalysis of ATP dependent homologous DNA pairing and strand exchange by the yeast RAD51 protein. Science 265:1241-1243.

Sung, P., and Robberson, D.L. 1995. DNA strand exchange mediated by RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. Cell. 82: 453-461.

Sung, P., Trujillo, K., and Van Komen, S. 2000. Recombination factors of Saccharomyces cerevisiae. Mutat. Res. 451: 257-275.

Swagemakers, S.M.A., Essers, J., de Wit, J., Hoeijmakers, J.H.J., and Kanaar, R. 1998. The human Rad54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase. J. Biol. Chem. 273: 28292-28297.

Trujillo KM, Yuan SS, Lee EY, Sung P. 1998. Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. J. Biol. Chem. 273: 21447-21450.

Van Komen, S., Petukhova, G., Sigurdsson, S., Stratton, S., and Sung, P. 2000. Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. Mol. Cell 6: 563-572.

Vignali, M., Hassan, A.H., Neely, K.E., and Workman, J.L. 2000. ATP-dependent chromatin-remodeling complexes. Mol. Cell. Biol. 20: 1899-1910.